IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Ingham et al.

Serial No: 08/462,386

Filed: June 5, 1995

For: Vertebrate Tissue Pattern Inducing Proteins and Uses Related Thereto

Art Unit: 1646

Attorney Docket No. HMSU-P04-006

Examiner: M. Brannock

Assistant Commissioner for Patents
Washington, DC 20231

Declaration Under 35 U.S.C. §1.132 of Hank Dudek

Sir:

I, Hank Dudek of Massachusetts, hereby declare as follows:

1. I am an employee of Curis Inc., which licenses the above-described application from Harvard Medical School, the assignee of record.

2. Experiments were performed in collaboration with me, the results of which are depicted in Exhibits A-F, which demonstrate the effects of treating adult tissues with *sonic hedgehog* or *desert hedgehog*. Exhibit A shows that both *sonic hedgehog* and *desert hedgehog* induce expression of *gli-1* in endoneurial fibroblasts isolated from adult rat sciatic nerve. Exhibits B and C show that both *sonic hedgehog* and *desert hedgehog* improve recovery in adult mice following sciatic nerve crush. Exhibit D shows that striatal administration of *sonic hedgehog* induces *gli-1* in adult rats. Exhibits E and F show that *sonic hedgehog* attenuates malonate-induced lesions in adult rat. Each of these experiments demonstrates that *hedgehog* treatment affects adult tissues.

3. The above experiments were performed in accord with the teachings of the abovementioned patent application.

4. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are

punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Hank Dudek

Dated: 12/13/01

Signature: Hank Dudek
Hank Dudek

Dhh and Shh Induce *gli-1* expression in Endoneurial Fibroblasts Isolated From Adult Rat Sciatic Nerve

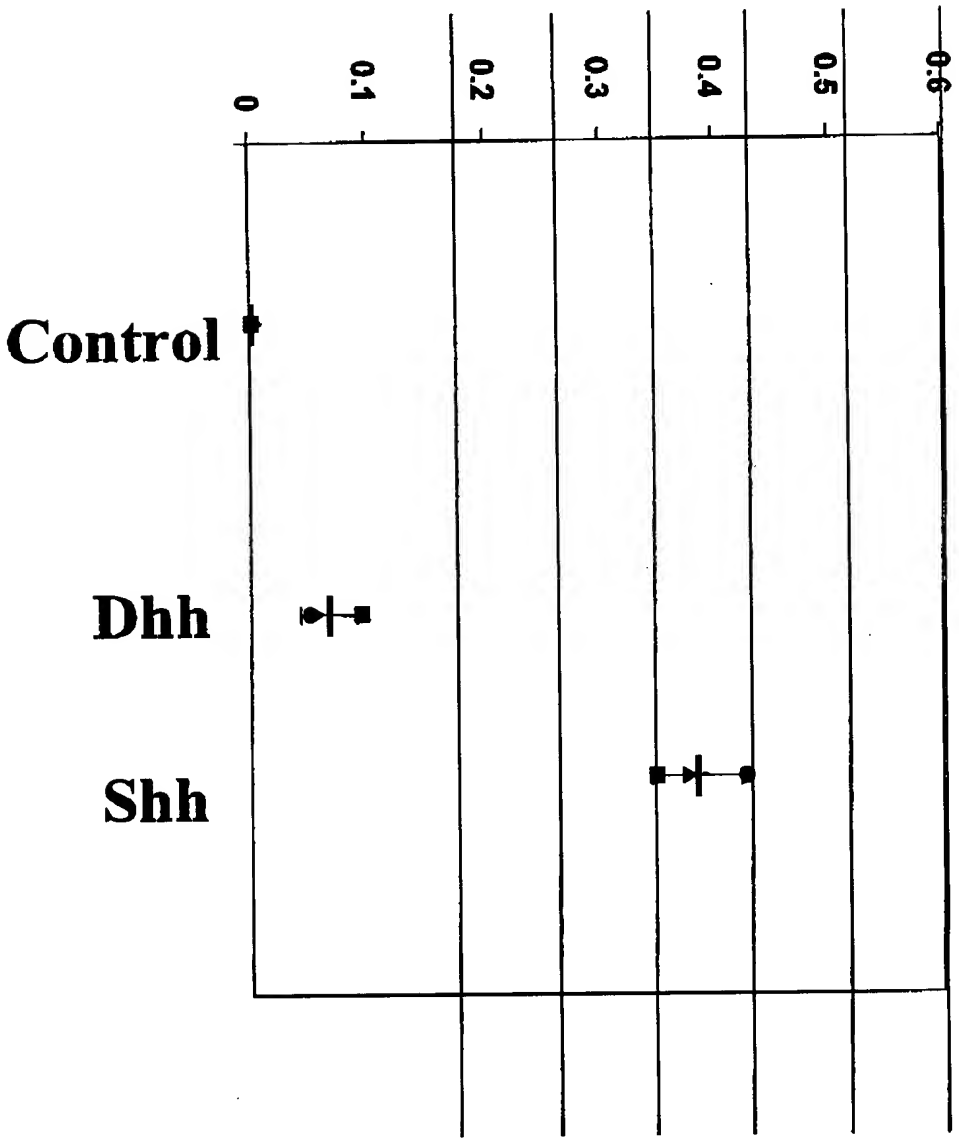


EXHIBIT A

Shh and Dhh Improve Recovery After Sciatic Nerve Crush in Adult Mice

EXHIBIT B

Ability to Grip

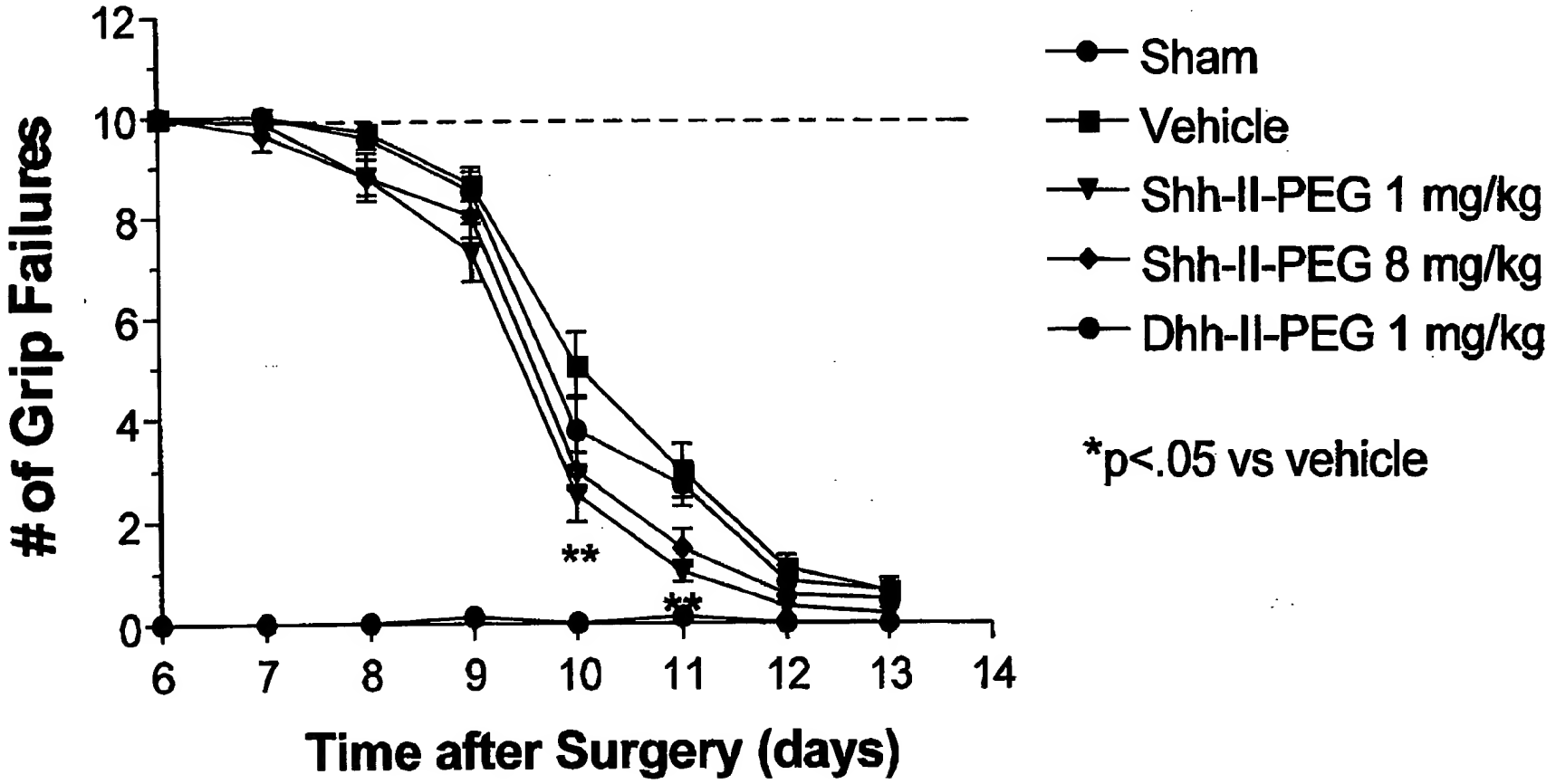
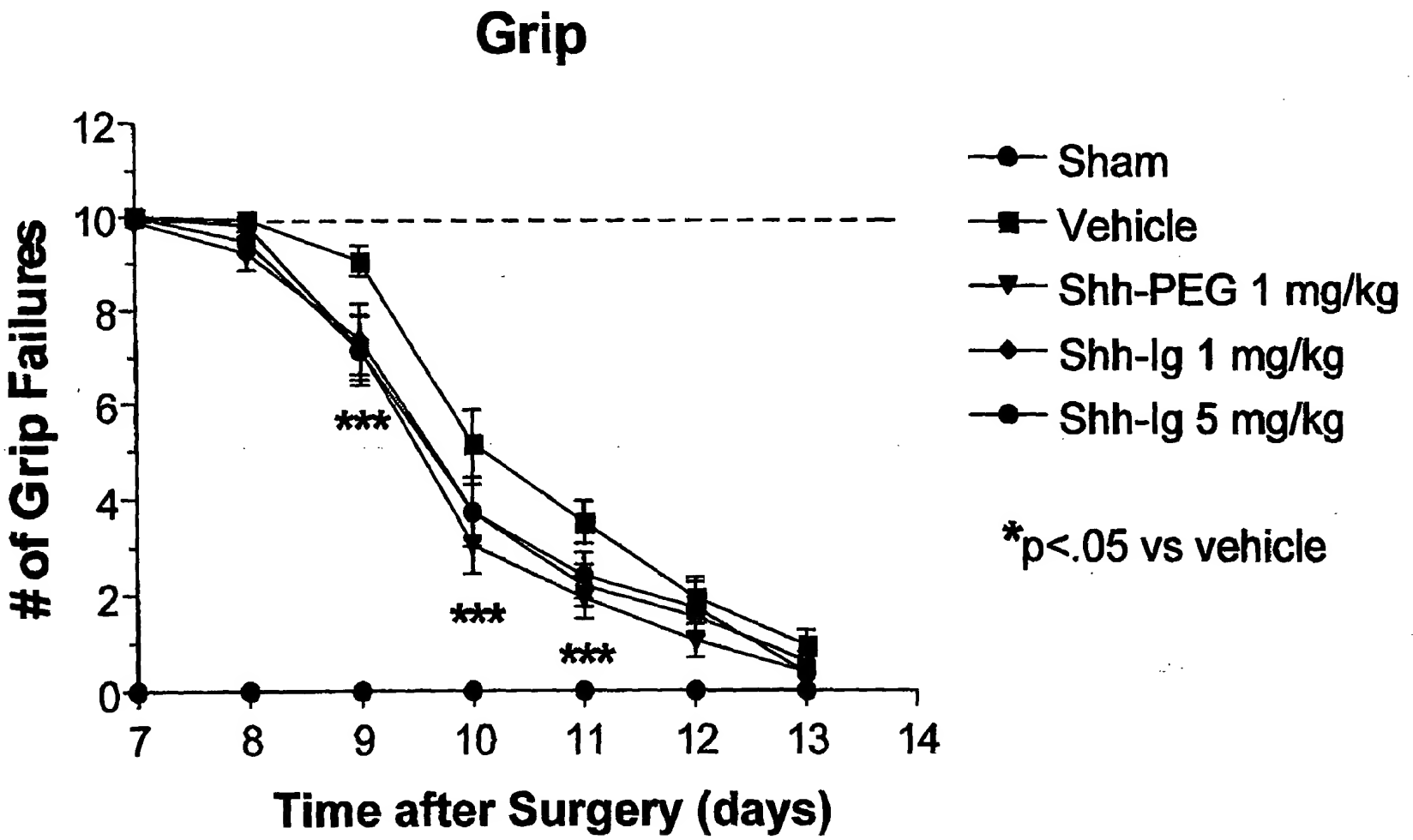


EXHIBIT C

Shh Improves Recovery After Sciatic Nerve Crush in Adult Mice



Striatal Administration of Shh Induces *gli-1* in Adult Rats

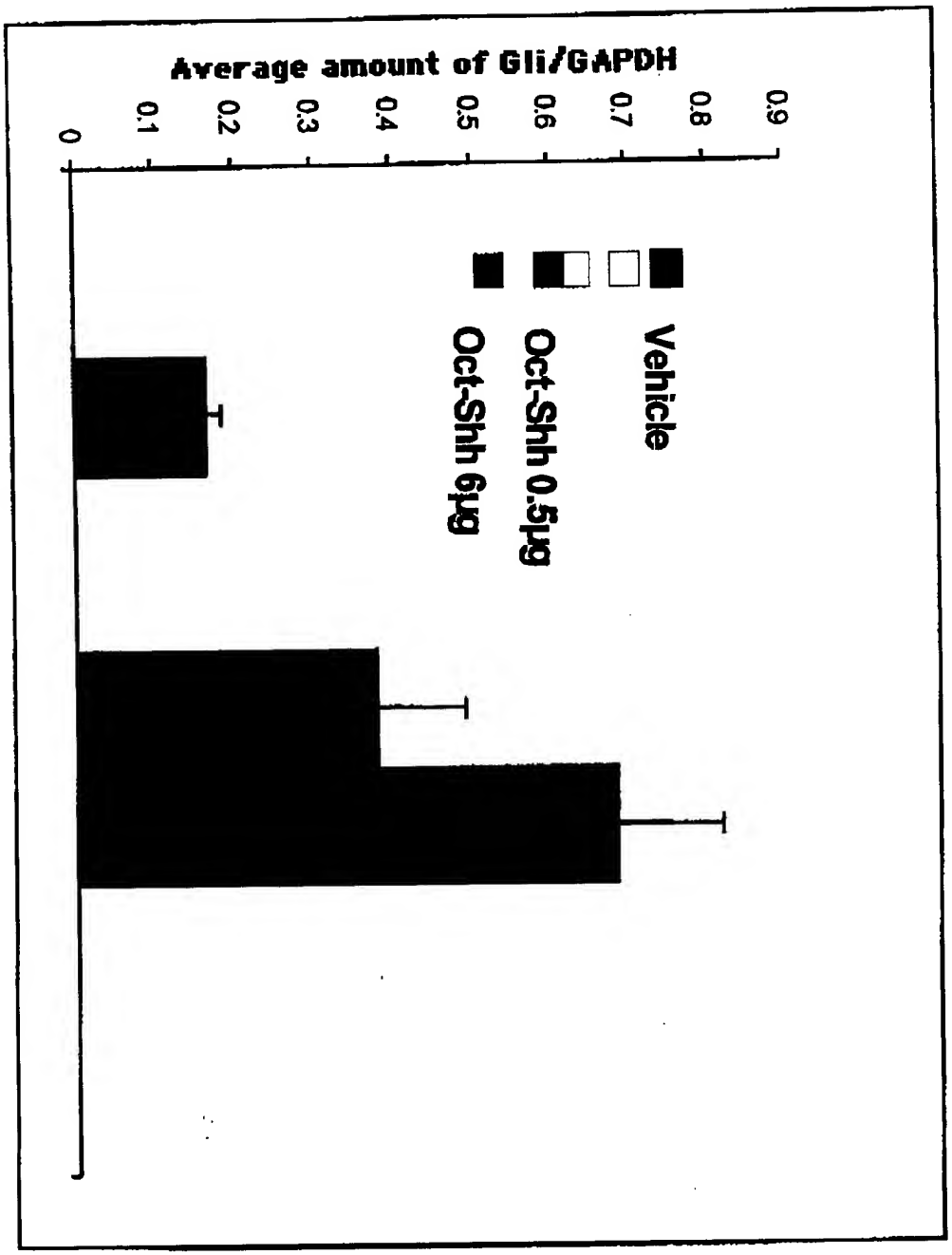


EXHIBIT D

Striatal Administration of II-Shh Attenuates Malonate-Induced Lesion in Adult Rats

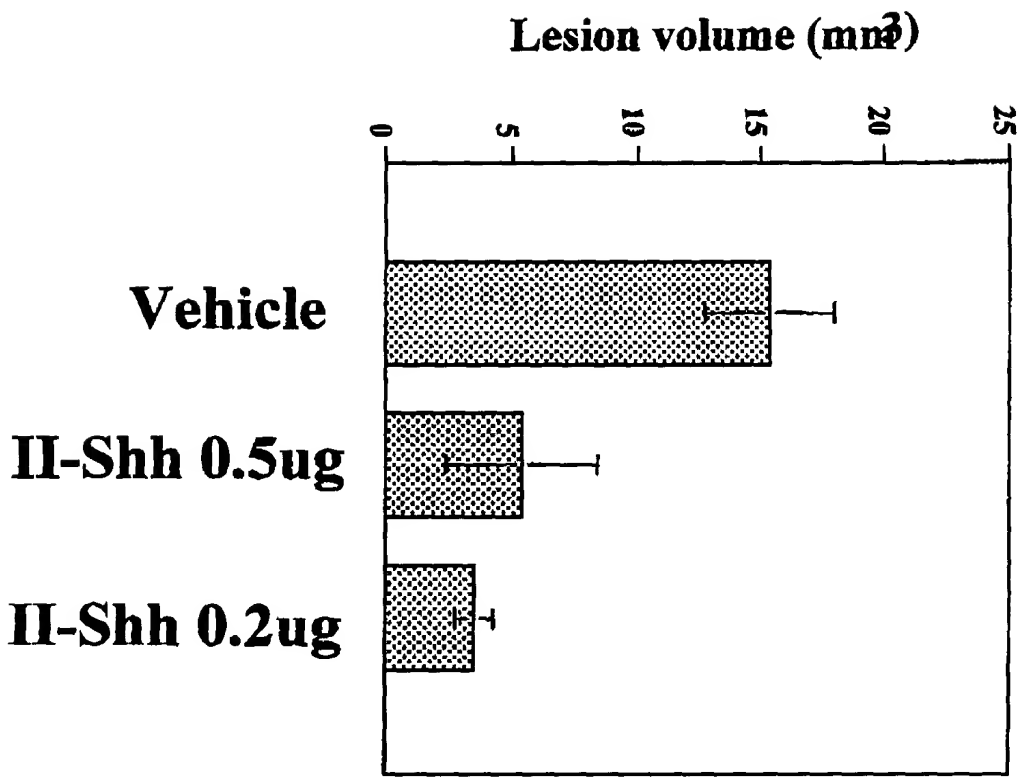


EXHIBIT E

Striatal Administration of Shh Attenuates Malonate-Induced Lesion in Adult Rats

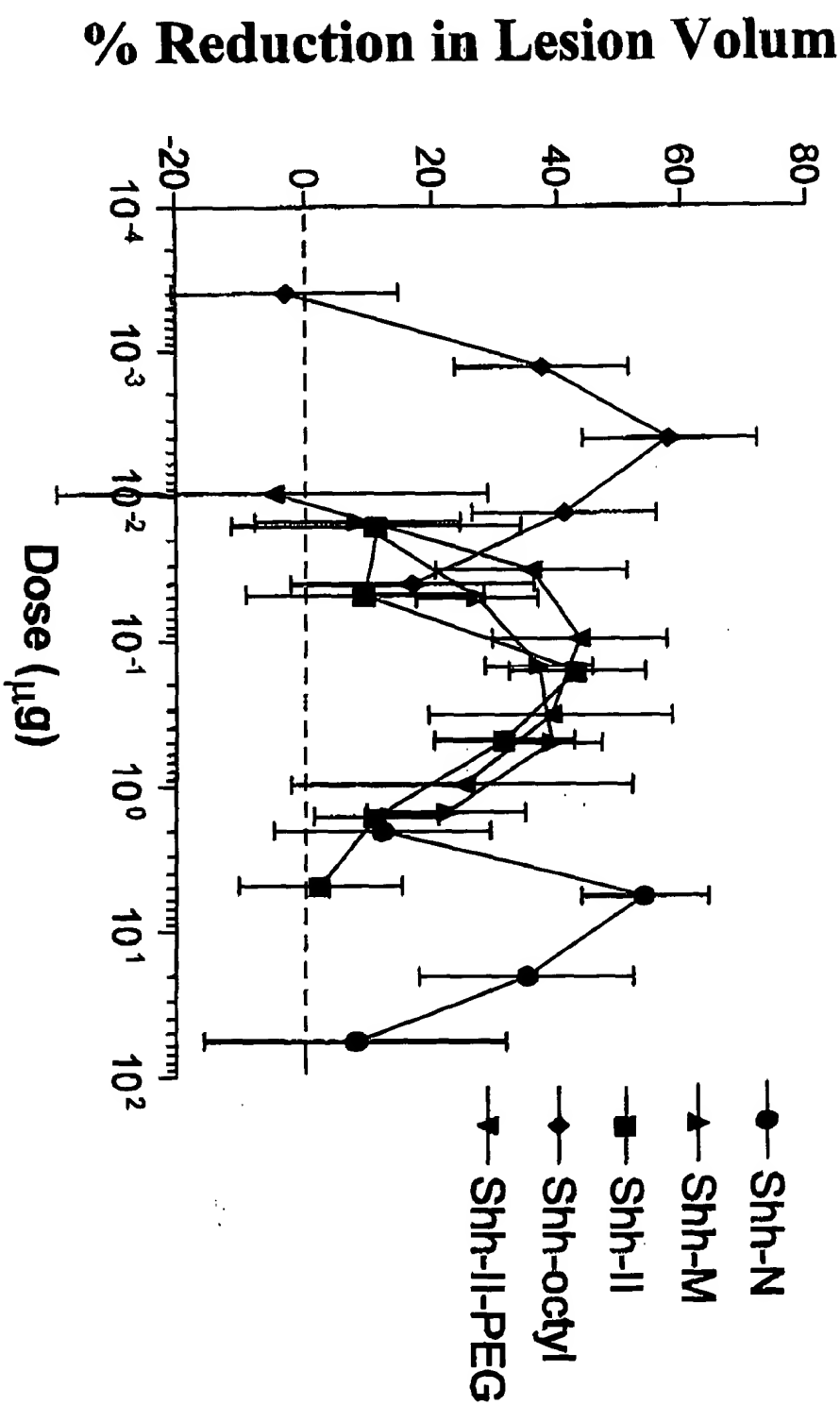


EXHIBIT C

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Comparative biological responses to human Sonic, Indian, and Desert hedgehog

Suji Pathi[a,1], Sylvia Pagan-Westphal[a,1], Darren P. Baker[b], Ellen A. Garber[b], Paul Rayhorn[b], David Bumcrot[c], Clifford J. Tabin[a], R. Blake Pepinsky[b], Kevin P. Williams[b,*]

[a]*Department of Genetics, Harvard Medical School, Boston, MA, USA*
[b]*Biogen Inc., 14 Cambridge Center, Cambridge, MA, USA*
[c]*Curis Inc., 45 Moulton Street, Cambridge, MA, USA*

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Abstract

A comprehensive comparison of Sonic (Shh), Indian (Ihh), and Desert (Dhh) hedgehog biological activities has not previously been undertaken. To test whether the three higher vertebrate Hh proteins have distinct biological properties, we compared recombinant forms of the N-terminal domains of human Shh, Ihh, and Dhh in a variety of cell-based and tissue explant assays in which their activities could be assessed at a range of concentrations. While we observed that the proteins were similar in their affinities for the Hh-binding proteins; Patched (Ptc) and Hedgehog-interacting protein (Hip), and were equipotent in their ability to induce Islet-1 in chick neural plate explant; there were dramatic differences in their potencies in several other assays. Most dramatic were the Hh-dependent responses of C3H10T1/2 cells, where relative potencies ranged from 80 nM for Shh, to 500 nM for Ihh, to >5 μM for Dhh. Similar trends in potency were seen in the ability of the three Hh protoins to induce differentiation of chondrocytes in embryonic mouse limbs, and to induce the expression of *nodal* in the lateral plate mesoderm of early chick embryos. However, in a chick embryo digit duplication assay used to measure polarizing activity, Ihh was the least active, and Dhh was almost as potent as Shh. These findings suggest that a mechanism for fine-tuning the biological actions of Shh, Ihh, and Dhh, exists beyond the simple temporal and spatial control of their expression domains within the developing and adult organism.

Keywords: Sonic; Indian; Desert; Hedgehog; Patched; C3H10T1/2; Nodal; Digit duplication; Chondrocyte differentiation

1. Introduction

Hedgehog (Hh) proteins are involved in many important developmental processes in vertebrates and invertebrates. In contrast to the single *hh* gene present in *Drospohila*, higher vertebrates have three *hh* genes, *Sonic* (*Shh*), *Indian* (*Ihh*), and *Desert* (*Dhh*) *hedgehog*, and additional family members are present in zebrafish and Xenopus. Each of the vertebrate *hh* appears to have a unique set of roles as first suggested by their distinct expression domains (Bitgood and McMahon, 1995), and subsequently by various functional studies, reviewed in Hammerschmidt et al. (1997). Whether these genes also encode proteins with distinct bioloogical properties is unknown. Shh, of the higher vertebrates homologs, is the most well-studied and has been implicated in the establishment of the early left–right (L–R) axis in the chick embryo (Levin et al., 1995; Tsukui et al., 1999), the regulation of ventral cell fates in the central nervous system (CNS) (Echelard et al., 1993; Ericson et al., 1996, 1997; Roelink et al., 1994, 1995), and the specification of antero-posterior (A-P) axis in the limb (Riddle et al., 1993). Ihh has been implicated in modulating chondrogenesis in the appendicular skeleton, and acts as a negative regulator of the differentiation of proliferating chondrocytes (Vortkamp et al., 1996). Dhh is implicated in germ-cell proliferation, the development of germ cells toward the later stages of spermatogenesis, in nerve–Schwann cell interactions (Bitgood and McMahon, 1995), and in signaling peripheral nerve ensheathment (Parmantier et al., 1999). Mice null for Shh (Chiang et al., 1996), Ihh (St.-Jacques et al., 1999), and Dhh (Bitgood et al., 1996) have further defined key developmental roles.

The mature amino terminal domain of Hh (Bumcrot et al., 1995; Lee et al., 1994) has been shown to be sufficient for all the known long- and short-range activities of this protein. Further biochemical studies have shown that Shh is modi-

* Corresponding author. Tel.: +1-617-679-3341; fax: +1-617-679-3148.
E-mail address: kevin_williams@biogen.com (K.P. Williams).
[1] S. Pathi and S. Pagan-Westphal contributed equally to this article.

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EXHIBIT 1

fied with a C-terminal cholesterol moiety (Porter et al., 1996), and an N-terminal palmitic acid group (Pepinsky et al., 1998) that serve to tether the protein to the cell surface of the expressing cell resulting in a lipid-modified Hh protein that is 30-fold more potent than the unmodified protein (Pepinsky et al., 1998). While short-range effects can be mediated by such a tethered protein, the mechanism by which this tethered signal is released or transported to mediate long-range effects is currently unclear, although a number of mechanisms have been proposed (reviewed in Christian, 2000; Chuang and Kornberg, 2000).

Vertebrate Hh proteins appear to be processed by the same mechanism (reviewed in Johnston and Scott, 1998) and use the same receptors and signal transduction pathway (Hammerschmidt et al., 1996; Johnston and Scott, 1998; Carpenter et al., 1998). The transmembrane protein Patched (Ptc) is required for cellular responsiveness to Hh, and is highly expressed in all cells known to be actively responding to any of the vertebrate Hh proteins, reviewed in Tabin and McMahon (1997). Ptc is also a receptor for the Hh protein (Ingham et al., 1991; Marigo et al., 1995; Stone et al., 1996). In the absence of Hh, Ptc represses the signaling activity of a second transmembrane protein Smoothened (Smo) (Alcedo et al., 1996; van den Heuvel and Ingham, 1996) by indirectly destabilizing Smo (Denef et al., 2000). Hh binding to Ptc causes Ptc to be removed from the cell surface (Denef et al., 2000), directing it to a distinct subcellular compartment and thereby relieving the repression of Smo signaling. Ptc itself is also an important transcriptional target of Hh signaling, activated downstream of Smo. Because of its ability to bind to Hh, the high levels of Ptc induced by Hh have the effect of acting as a sink, binding additional Hh molecules and thus limiting the range of Hh action (Chen and Struhl, 1996). Vertebrates have at least two *Ptc* genes: *Ptc* (also referred to as *Ptc-1*) and *Ptc-2* (Goodrich et al., 1996; Marigo et al., 1996; Motoyama et al., 1998; Takabatake et al., 1997). Vertebrates also have another Hh-binding protein, hedgehog-interacting protein (Hip), which is also inducible by Hh (Chuang and McMahon, 1999). Zinc-finger transcription factors of the *Gli* family also play critical roles in mediating Hh signaling (reviewed in Ruiz i Altaba, 1999).

A number of assays have been established to assess the effects of the Hh proteins in vitro and in vivo including those assessing alkaline phosphatase induction in C3H10T1/2 cells (Day et al., 1999; Katsuura et al., 1999; Kinto et al., 1997; Nakamura et al., 1997; Pepinsky et al., 1998; Williams et al., 1999), lateralizing activity (Levin et al., 1995; Pagan-Westphal and Tabin, 1998), digit duplication (Riddle et al., 1993; Wada et al., 1999; Yang et al., 1997), motor neuron induction (Roelink et al., 1995), and chondrocyte proliferation (Vortkamp et al., 1996). To determine whether the three forms of vertebrate Hh have an equivalent capacity to induce specific biological responses in vivo, we compared the human forms of Shh, Ihh, and Dhh in these assays over a range of concentrations. We find that all three proteins can elicit similar biological responses, but that their relative potencies differ in an assay-dependent manner.

2. Results

2.1. Structural and functional characterization of human Shh, Ihh, and Dhh

The mature forms of human Shh, Ihh, and Dhh, encoding residues 24–197, residues 28–202, and residues 23–198, respectively (Fig. 1), were expressed in *E. coli* and purified by conventional chromatography. The recombinant proteins were tested for their ability to bind the Hh-binding proteins Ptc and Hip: Shh, Ihh, and Dhh bound Ptc with comparable IC_{50} values, in the 3–7 nM range (Fig. 2) and bound Hip with comparable IC_{50} values, in the 6–15 nM range. Structural characteristics of Shh, Ihh, and Dhh were assessed by thermal denaturation using circular dichroism as a measure of secondary and tertiary structures. The proteins all showed cooperative unfolding and gave comparable T_m values (Shh = 58°C, Ihh = 58°C, and Dhh = 57°C). These analyses indicate that the recombinant proteins are functional and have similar structure.

2.2. Comparative assessment of human Shh, Ihh, and Dhh in cell-based assays

Shh, Ihh, and Dhh were next subjected to analysis in a number of Hh-responsive cell lines. The Hh-responsive cell line C3H10T1/2 has been used to assess the activity of a range of Hh protein forms (Day et al., 1999; Katsuura et al., 1999; Kinto et al., 1997; Nakamura et al., 1997; Pepinsky t al., 1998; Williams et al., 1999). Human Shh produced a dose-dependent response in the C3H10T1/2 alkaline phosphatase (AP) induction assay, with an EC_{50} of 80 nM (Fig. 3A) consistent with our previous observations (Pepinsky et al., 1998; Williams et al., 1999). In the same assay, recom-

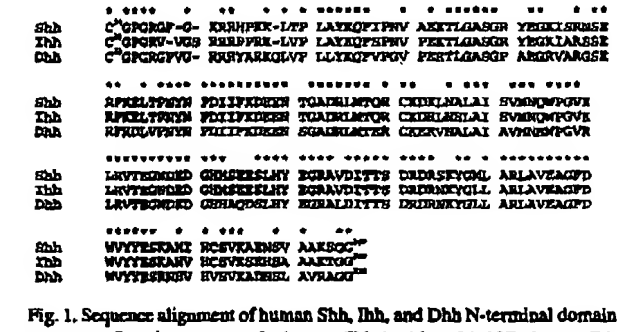

Fig. 1. Sequence alignment of human Shh, Ihh, and Dhh N-terminal domain sequences. Protein sequences for human Shh (residues 24–197), human Ihh (residues 28–202) and human Dhh (residues 23–198) are shown. Gaps introduced to facilitate alignment are represented by dashes. Residues conserved in all three proteins are shown with asterisks above. Shh has 91% identity with Ihh (one extra residue in Ihh + 17 amino acid differences); Shh has 76% identity with Dhh (two extra residues in Dhh + 42 amino acid differences); Ihh has 80% identity with Dhh (one extra residue in Ihh + 36 amino acid differences).

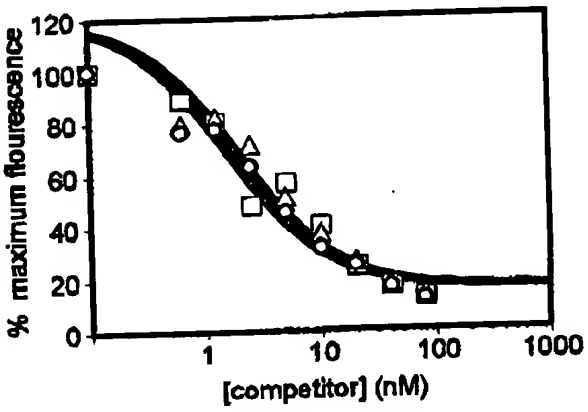

Fig. 2. Comparison of human Shh, Ihh, and Dhh binding to Ptc. The relative potency of human Shh (□), Ihh (Δ), and Dhh (○) for binding to Ptc was assessed on *ptc*-transfected EBNA-293 cells by FACS analysis. Serial dilutions of the test samples were incubated with the *ptc*-transfected EBNA-293 cells and washed, and then the percent binding was measured by the ability of the proteins to compete with Shh-Fc for binding to the cells. Bound Shh-Fc was quantified by mean fluorescence. The data were fitted to a hyperbolic curve by non-linear regression.

binant human Ihh and Dhh have lower potencies (Fig. 3A) with EC_{50} values of 500 nM and >5 μM, respectively, and corresponding lower efficacies, as assessed by the maximal AP response. Despite the large differences in potency, the three Hh proteins exhibited similar induction time courses, with maximal AP response at 5 days (data not shown). Furthermore, these differences in potency could not be accounted for by differences in solubility or stability in the assay medium (data not shown).

Since C3H10T1/2 is a mesenchymal stem cell line (Reznikoff et al., 1973) that can differentiate into a number of different lineages, including osteoblasts, chondrocytes, adipoycytes, and myoblasts, depending on the factor added and the conditions of incubation (Ibric et al., 1988; Katagiri et al., 1990; Taylor and Jones, 1979; Wang et al., 1993), we tested the possibility that Ihh and Dhh might induce differentiation into different cell types, thus accounting for the differences in AP responsiveness observed in this assay. However, when compared to Shh, the reduced potency of Ihh and Dhh for osteoblast induction (as assessed by histochemical detection of AP), was not reflected in the appearance of any other cell types such as adipocytes (as detected by Oil red staining, data not shown). In turn, because the AP induction assay takes 5 days to develop a maximal response, another possibility was that the observed differences in potency might reflect differences in downstream AP signaling events rather than more upstream Hh signaling events. To test this possibility, we assayed Shh, Ihh, and Dhh in C3H10T1/2 cells transfected with a Hh-responsive promoter-luciferase construct, *gli-luc*, where the Hh signaling response is coupled to activation f a known Hh-responsive gene (*gli-1*) and maximal activity is observed within 1 day by measuring luciferase activity. In the 18-h C3H10T1/2 *gli-luc* assay, we observed the same rank and range of potencies for Shh, Ihh, and Dhh as in the AP induction assay, with EC_{50} values of 80 nM, 500 nM, and 5 μM, respectively, suggesting that the differences in potency are at the level of Hh signaling and not further downstream. As an independent measure of Hh-dependant signaling, we also evaluated the ability of the Hh proteins to induce *gli-1*, *ptc*, *ptc-2*, and *hip* mRNA using RT-PCR assays in C3H10T1/2 cells. Shh, Ihh, and Dhh had the same dose responses for each gene, with Shh being more potent than Ihh with Dhh the weakest. Representative data for *gli-1* mRNA induction, as measured by RT-PCR, are shown in Fig. 3B. These findings were confirmed by quantitative PCR studies (data not shown).

2.3. The effects of Ihh, Dhh, and Shh on chondrocyte differentiation in cultured mouse limbs

When proliferating chondrocytes commit to the differentiation pathway, they transiently express Ihh as pre-hypertrophic cells, which leads to a signaling cascade that prevents further cells from entering the pathway toward hypertrophy. This has been demonstrated in vivo, but can also be recapitulated in vitro by directly adding Ihh protein to bone explant cultures (Vortkamp et al., 1996). We compared the relative potencies of the three Hh proteins

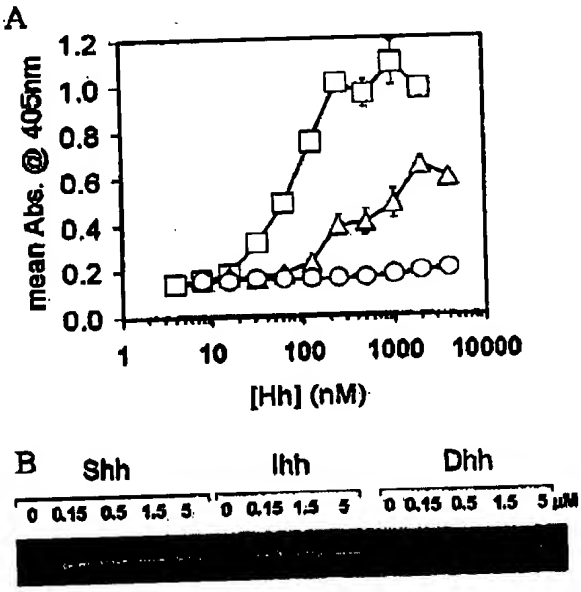

Fig. 3. Human Shh, Ihh, and Dhh have different potencies on C3H10T1/2 cells. (A) The relative activities of Shh, Ihh, and Dhh were assessed in the C3H10T1/2 AP induction assay. Serial two-fold dilutions of Shh (□), Ihh (Δ), and Dhh (○) were incubated with the cells for 5 days and the resulting levels of AP activity measured at 405 nm using the AP chromogenic substrate *p*-nitrophenyl phosphate. (B) Induction of *gli-1* mRNA by Shh, Ihh, and Dhh in C3H10T1/2 cells. RT-PCR for *gli-1* was carried out on total RNA extracted from C3H10T1/2 cells that were incubated for 5 days with the indicated concentrations of Shh, Ihh, and Dhh. Loadings were normalized to actin levels.

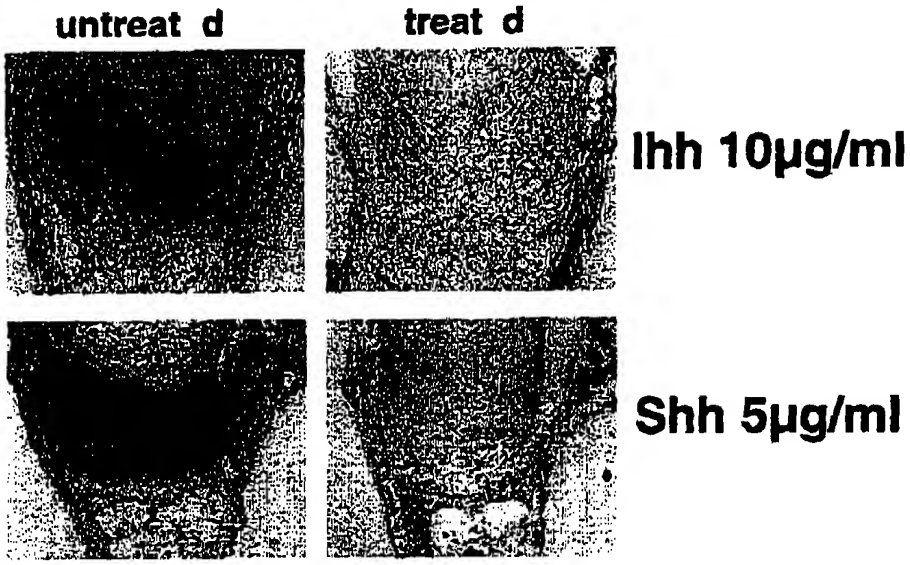

Fig. 4. Examples of total suppression of *Collagen X* expression. E16.5 mouse proximal tibial growth plates were hybridized to a mouse *Collagen X* riboprobe. The examples shown are the lowest concentrations at which Ihh and Shh can completely suppress hypertrophic differentiation of chondrocytes. Treated limbs show a near absence in the expression of *Collagen X* as displayed by the lack of silver grains above background levels. Each protein and concentration was tested in five independent cultures. Dhh was not able to fully suppress *Collagen X* expression at any of the protein concentrations tested.

in suppressing hypertrophic chondrocyte differentiation, a role played by Ihh during normal bone development. Shh, Ihh, and Dhh proteins were added to E16.5 mouse tibia organ cultures as described in Section 4 (Experimental procedures). Section in situ hybridization of the treated and untreated contralateral control limbs revealed differences in the ability of the three proteins to suppress the hypertrophic differentiation of chondrocytes, as measured by the marker for hypertrophic chondrocytes, *Collagen X*. The activities of the three Hh proteins were compared in two ways by determining (i) the lowest concentration at which the exogenous protein totally suppresses *Collagen X* expression (Fig. 4), and (ii) the lowest concentration at which the exogenous protein causes a difference between the treated and untreated contralateral limbs as measured by decreased density of *Collagen X* expression (Fig. 5). Shh completely suppressed *Collagen X* expression at a concentration of 5 µg/ml (Fig. 4). At this concentration, Ihh was not as effective; however, there was a decrease in the amount and density of silver grains in the treated limbs as compared to the untreated contralateral control (data not shown). With Ihh, near total suppression of *Collagen X* expression was achieved at a concentration of 10 µg/ml (Fig. 4), Dhh was unable to fully suppress *Collagen X* expression at any of the protein concentrations tested. The lowest Shh concentration at which treated limbs were noticeably different from their contralateral controls, in terms of *Collagen X* expression, was 0.5 µg/ml (Fig. 5). At this concentration, the Shh-treated limbs showed a decreased density of silver grains as compared to the controls. Ihh and Dhh consistently showed less dense *Collagen X* expression at concentrations of 5 and 20 µg/ml, respectively (Fig. 5). Thus, in this in vitro organ culture assay, Shh appears to be the most potent suppressor

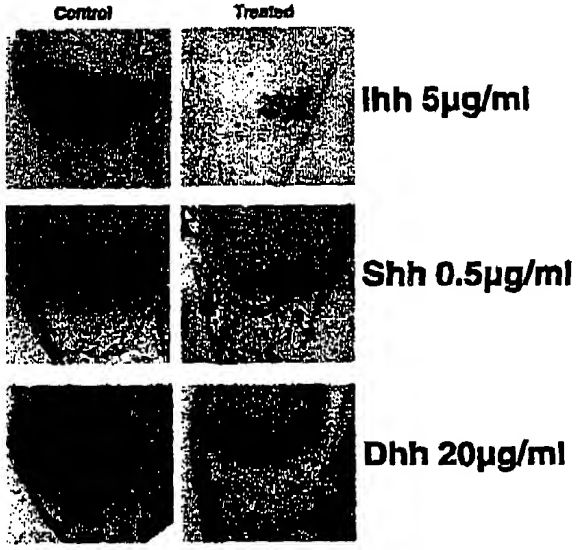

Fig. 5. Examples of cultures showing decreased density of *Collagen X* expression. E16.5 mouse proximal tibial growth plates were hybridized to a mouse *Collagen X* riboprobe. Treated limbs show a decrease in the expression of *Collagen X* as displayed by the decreased density of silver grains. Examples shown correspond to the lowest concentration where a reproducible effect was seen for each. Limbs treated with lower concentrations of their respective proteins have virtually no discernible differences from their untreated contralateral controls. Each protein and concentration was tested in five independent cultures.

of chondrocyte hypertrophy, followed by Ihh, with Dhh having the weakest potency.

2.4. Lateralizing activity of human Shh, Ihh, and Dhh

We next compared the relative potencies of the three Hh proteins in assays based on the early embryonic roles of Shh. The earliest known function of Shh in the chick embryo is in the establishment of left–right asymmetry. Shh, produced exclusively by cells on the left side of Hensen's node, triggers a cascade of signals leading to the expression of several left-side-specific genes, including the transforming growth factor (TGF)-β family member *nodal*, in the lateral plate mesoderm (LPM) (Levin et al., 1995). This lateralizing activity can be assessed by adding Shh protein to the right side of the node, causing *nodal* to be ectopically induced on the right side and resulting in the randomizing of heart sidedness (Levin et al., 1995; Pagan-Westphal and Tabin, 1998). At Shh concentrations of 0.1 mg/ml or greater, *nodal* was activated in a domain identical to that normally seen on the left (Fig. 6A), while at lower concentrations (0.05 mg/ml), *nodal* was activated in a more limited domain and/or in a smaller percentage of embryos (Fig. 6B,C). We used this as an assay to test the relative ability of the different Hh proteins to induce the left-sided signaling cascade, scoring the percentage of embryos displaying any ectopic *nodal* expression following implantation of a protein-laden Affigel-Blue bead (Fig. 6D). While Shh induced *nodal* in 100% of the embryos at 1, 0.5, and 0.1 mg/ml, Ihh required at least 0.5 mg/ml to achieve this effect, only inducing *nodal* in 60% of the embryos at 0.1 mg/ml. Dhh was even less potent in this assay, inducing *nodal* in only 75% of the embryos at 0.5 mg/ml. Thus, the relative potencies of the Hh proteins at inducing the left-sided cascade was the same as in the chondrocyte differentiation, with Shh being the most potent, followed by Ihh, and then by Dhh.

2.5. Polarizing activity of human Shh, Ihh, and Dhh

We next compared the relative potencies of the Hh proteins in causing digit duplications in chick wing buds. In the vertebrate limb, Shh is responsible for A-P patterning as well as supporting the regulation of the proximo-distal (P-D) outgrowth of the limb. During the limb bud stages, Shh is expressed in a subset of posterior mesenchymal cells known as the zone of polarizing activity (ZPA) (Riddle et al., 1993). When Shh-expressing cells or beads containing Shh are implanted into the anterior portion of the limb, a mirror image duplication of the digits results (Riddle et al., 1993; Wada et al., 1999). The degree of digit duplication is Hh-concentration dependent and thus serves as an assay for Shh polarizing activity (Yang et al., 1997). Chicks have three wing digits, referred to as digits 4, 3, and 2 (from posterior to anterior). High levels of Shh ectopically released in the anterior causes a full mirror-image duplication, resulting in a 4-3-2-2-3-4 pattern. Lower levels of Shh result in a less complete duplication. Since a higher level of polarizing activity is required to duplicate a digit 4 than a digit 3, and still less is required to duplicate a digit 2, a quantitative score of polarizing activity can be assigned to a given limb skeletal pattern based on the types of ectopic digits that form (Fig. 7A). Different concentrations of each Hh protein were loaded onto Affigel-Blue beads (as used in the *nodal* induction assay above) and the data are shown in Fig. 7B. Shh showed strong polarizing activity, with an average score of four or higher at 8, 2, and 0.5 mg/ml. Dhh also produced duplications scored at four or higher at 8 and 2 mg/ml, but dropped down to a score of two at 0.5 mg/ml. In contrast, Ihh did not give rise to duplications with an average score above two even at 8 mg/ml. Thus, although the same batches of protein and same beads for delivery were used in the lateralizing and polarizing assays, the relative order of potency in the latter assay was different from the two previous assays for chondrocyte differentiation and lateralizing activity. Shh was still the most potent, but Dhh was significantly better than Ihh at eliciting digit duplications.

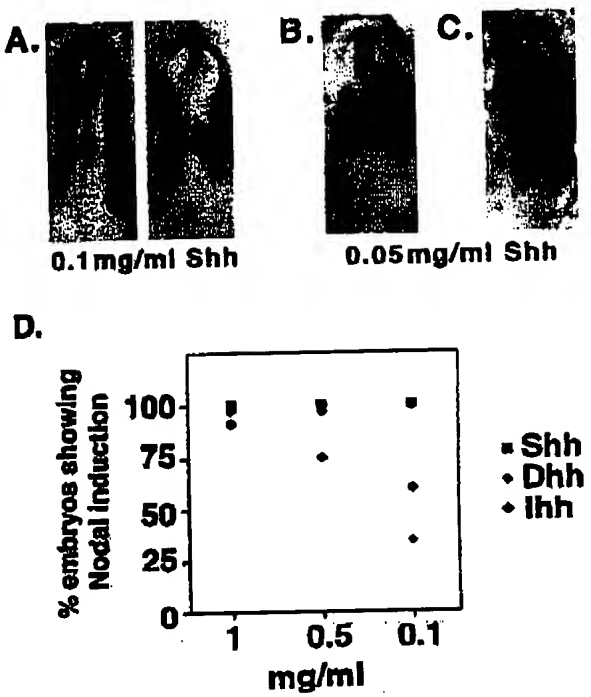

Fig. 6. *Nodal* induction in the right LPM in response to human Hh proteins. Beads soaked in Hh protein were implanted to the right of Hensen's node in stage 4 chick embryos in New culture. At stage 8, the embryos were harvested and *nodal* expression was examined by whole mount in situ hybridization. (A) In response to beads soaked in 0.1 mg/ml Shh, all embryos (two examples are shown) showed bilateral *nodal* expression. (B) At 0.05 mg/ml Shh, many embryos have only partial *nodal* induction, or (C) fail to show any *nodal* expression on the right side. (D) Percent *nodal* induction in the left LPM following Hh protein application. Affigel-Blue beads were soaked in the Hh protein concentrations shown and implanted into gastrulating chick embryos. Each symbol represents the percent *nodal* induction from 10 embryos. Shh (black ■); Ihh (green ●); Dhh (red ◆).

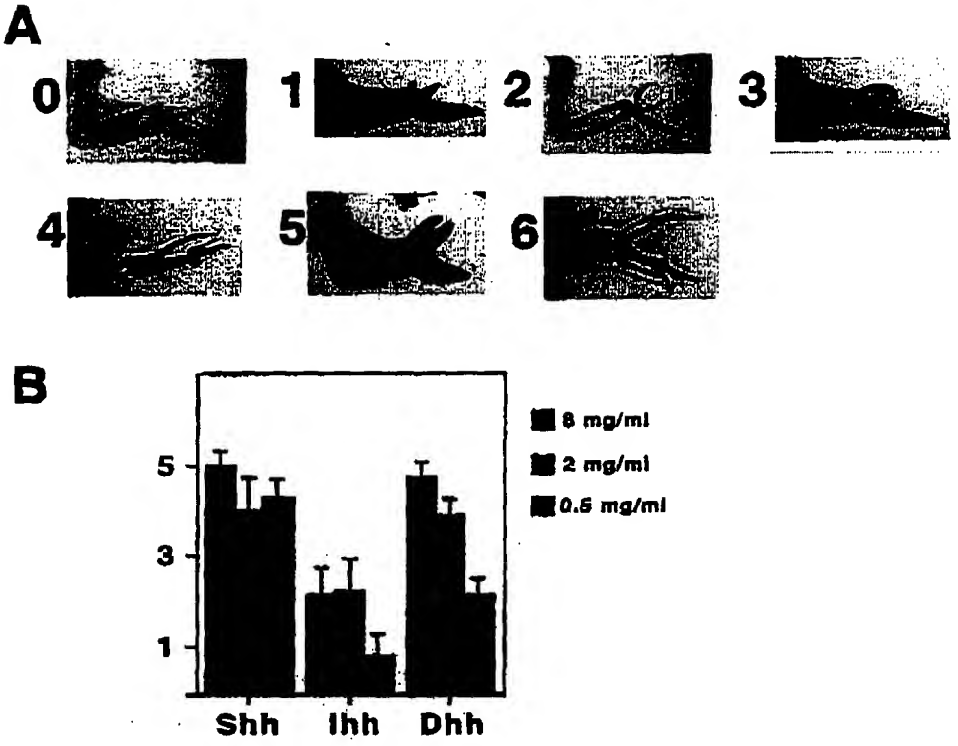

Fig. 7. Relative potencies of human Shh, Ihh, and Dhh in causing digit duplications in chick wing buds. (A) Assay for polarizing activity. Shh protein was loaded onto Affigel-Blue beads and implanted into stage 22 limb buds. In response to different concentrations of Shh protein, a variety of skeletal phenotypes are induced in the limb. A numerical score for the level of polarizing activity is assigned based on the type of ectopic digits produced (0 = no duplication; 1, a partial ectopic digit; 2, a full ectopic digit; 3, an ectopic digit 2 plus an additional partial digit; 4, an ectopic digit 3; 5, an ectopic digit 3 plus an additional partial digit; 6, an ectopic digit 4). Examples of each phenotype and the score assigned are shown. (B) Polarizing activity was scored on the six-point scale following implantation of Affigel-Blue beads soaked in hedgehog protein at 8 (red bar), 2 (blue bar) or 0.5 mg/ml (green bar). Average polarizing activity scores are shown.

2.6. *Motor neuron-inducing activity of Shh, Ihh, and Dhh proteins*

As an independent measure of function, we also tested the ability of each Hh protein to induce motor neurons in lateral neural tube explants. The induction of neural cell types in response to Shh secreted from the notochord and floor plate in vivo is dose-dependent and can be recapitulated in vitro with explants of lateral neural tube, which have not been exposed to Shh. The induction of motor neurons in such explants can be assayed by antibodies specific to the marker Islet-1 (Roelink et al., 1995). In this assay, Shh, Ihh, and Dhh were equipotent in their Islet-1-inducing activity, with an EC_{50} value of 2 nM.

3. Discussion

A comprehensive comparison of the biological activities of Shh, Ihh, and Dhh hedgehog has not previously been undertaken. To test whether the three higher vertebrate Hh proteins have distinct biological properties, we compared recombinant forms of the N-terminal domains of human Shh, Ihh, and Dhh in a variety of cell-based and tissue explant assays. The highly conserved sequences of Shh, Ihh, and Dhh (Fig. 1) would suggest that they could have overlapping or identical activities (Kumar et al., 1996; Shimeld, 1999). Thus, their activities might be expected to be similar to one another. However, the comparative data for all the assays, summarized in Table 1, does not support this notion. While the three proteins bind the receptor Ptc and Hip with similar affinity and were equipotent in their ability to induce Islet-1 in neural plate explants, in other in vitro assays, large differences in their potency was observed. The rank order of potency was generally Shh > Ihh > Dhh, with Shh and Ihh more closely related in terms of potency than Shh and Dhh, or Ihh and Dhh. Only in the digit duplication assay was the rank order of potencies different; Shh was again the most potent, but in this assay, Dhh was more potent than Ihh. Previous studies have shown that Ihh at sufficient dosage can cause digit duplications (Yang et al., 1998). One possible reason that Ihh may not have been as effective as Shh or Dhh at inducing digit duplication is that its activity was manifested in additional effects such as

Table 1
Differences in the responsiveness of biological systems to human Shh, Ihh, and Dhh

Protein*	Shh	Ihh	Dhh
Ptc binding (IC_{50})	3.7 nM	7.1 nM	6.8 nM
Hip binding (IC_{50})	6 nM	15 nM	9 nM
C3H10T1/2 AP induction (EC_{50})	80 nM	500 nM	> 5 μM
C3H10T1/2 gli-luc induction (EC_{50})	80 nM	500 nM	> 5 μM
TM3 gli-luc induction (EC_{50})	80 nM	320 nM	800 nM
Islet-1 induction (EC_{50})	2 nM	2 nM	2 nM
Chondrocyte differentiation	++++	++	+
Left/right nodal induction	++++	++	+
Digit duplication	++++	+	+++

* Proteins were generated and assays carried out as described in Section 4 (Experimental procedures).

increasing the thickness of the bones (data not shown). However, even in systems that might be expected to be Ihh-responsive, such as those involved in bone development (chondrocyte differentiation and osteoblast induction in C3H10T1/2 cells), Shh was still the most potent of the three Hh proteins and had an even greater effect on bone thickness than Ihh. It is unclear whether the quantitative differences seen here for the three human Hh proteins are a reflection of differing biological activities, of unidentified components that can modulate their responses dependent on the context of the assay system or from sequence differences for the different proteins.

Nevertheless, there is evidence from zebrafish that under physiological conditions, not all Hh proteins function equivalently. In particular, the zebrafish genes *echidna hedgehog* (*Ehh*), an Ihh homolog, and *Shh*, have been shown to have distinct biological roles: induction of muscle pioneer (MP) cells specifically requires *Ehh* in conjunction with *Shh* (Currie and Ingham, 1996), and micro-injection of *Ehh*, but not *Shh*, can restore MP cells in mutants which have a disrupted notochord. This strongly indicates that the two proteins have distinct biological activities. Conversely, *netrin-1a* expression in zebrafish somites is induced by *Shh* but not by *Ehh* (Lauderdale et al., 1998). We do not know whether these differences are a consequence of true independent activities, or whether it is an assay-dependent effect. It would be interesting to assay the purified zebrafish Ehh and Shh proteins in vitro to determine whether they have overlapping or independent activities.

The differences in potency of human Shh, Ihh, and Dhh described in this study (Table 1) are unlikely to be a consequence of differences in the structural integrity of the three Hh proteins, as they all appear to be folded similarly and can bind the receptor Ptc and Hip with comparable affinities (Table 1). Published studies (Carpenter et al., 1998) demonstrate that in addition to Hip and Ptc, all three mammalian Hh proteins bind with comparable affinities to Ptc-2. Moreover, we also found that Shh, Ihh, and Dhh had comparable activities in the neural plate explant assay (Table 1), indicating that the recombinant forms of Ihh and Dhh can be as functional as Shh in at least one biological system.

Previously, we observed that N-terminally truncated (Williams et al., 1999) and lipid-modified (Pepinsky et al., 1998) forms of Shh bound Ptc with comparable affinities and induced Islet-1 with comparable potencies, but had a wide range of potencies in the C3H10T1/2 assay, with the truncated form being inactive and the lipid-modified form being 30-fold more potent than the unmodified protein. In addition, we found that the activity of Shh in C3H10T1/2 cells, but not in the neural plate explant or Ptc-binding assays, was highly dependent on the N-terminal region of the protein (Williams et al., 1999).

To further investigate whether the differences in relative potencies of the three Hh proteins could be ascribed to effects mediated by the N-terminal region, we have generated chimeras of Shh and Dhh with the first 10 residues of the N-terminal domains swapped (K.P. Williams, E.A. Garber, and P. Rayhorn, unpublished data). Substituting the first 10 residues of Shh with those from Dhh substantially reduced the activity of this chimera (EC_{50} = 400 nM) compared to wild type Shh (EC_{50} = 80 nM), confirming the importance of the N-terminal region of Shh for activity in the C3H10T1/2 assay. The reverse experiment in which we replaced the first 10 residues of Dhh with those of Shh led to a modest increase in potency of this chimera (EC_{50} = 2 μM) compared to wild type Dhh (EC_{50} > 5 μM), suggesting that the inability to regain full activity is not simply due to differences in sequence in this region. These changes in activity are probably not due to gross changes in structure at the N-terminus, since in the X-ray structure of Shh (Pepinsky et al., 2000), this region is extended from the core and is presumably not important for the correct folding of the core protein. Furthermore, circular dichroism of wild type Shh and the N-terminally truncated form of Shh had comparable spectra (Williams et al., 1999). Although the N-terminal portion of the Hh genes are highly conserved in particular the first five amino acid residues which are absolutely conserved in all Hh proteins found to date (Kumar et al., 1996); there is an additional residue for Dhh within the N-terminal region which may result in spatial differences in this region.

An additional observation that we have made in the course of purifying these proteins is that all three Hh proteins bind to heparin. Significantly, Shh- and Ihh-binding were comparable, whereas Dhh appeared to have a much tighter interaction, as assessed by the sodium chloride concentration required for elution. In this regard, one intriguing possibility is that differences in the N-terminal region for the three Hh proteins, though not affecting their ability to bind Ptc, affect their interactions with accessory molecules on the cell surface that in turn modulate their activity. A role for accessory molecules in mediating Hh activity has been implicated previously, particularly the role of proteoglycans in mediating Hh long-range signaling, alth ugh a number of

other mechanisms have been proposed, including those mediated by lipid rafts (reviewed in Christian, .2000; Chuang and Kornberg, 2000) and by the Hh receptors themselves (Incardona et al., 2000). Although Shh, Ihh, and Dhh bind Ptc, Ptc-2, and Hip with comparable affinities when the receptors are overexpressed on cells, it may be that in their natural settings these binding events are modified by additional interactions with accessory molecules. Such differences in cellular context may explain the differences in potency of Dhh in C3H10T1/2 cells versus the Leydig cell line TM3, where we observed a substantial increase in the potency of Dhh compared to its potency in C3H10T1/2 cells, although it was still less potent than Shh or Ihh (Table 1). Significantly, Dhh is expressed in Sertoli cells and Leydig cells have been identified as the responding cell type (Bitgood et al., 1996; Clark et al., 2000). Furthermore, Ptc-2, but not Ptc, has been localized to Leydig cells, and Ptc-2 has been proposed to be the endogenous receptor for Dhh (Carpenter et al., 1998). Thus, in TM3 cells with Ptc-2 but not Ptc present, the repertoire of accessory molecules on these cells may act to increase the interaction of Dhh with Ptc-2.

The multiple *hh* genes of vertebrates have presumably arisen by duplication and subsequent divergence of a single ancestral *hh* gene. Although Shh, Ihh, and Dhh are highly homologous, Shh is much closer to Ihh than Dhh in sequence identity (Fig. 1). Our findings in a range of biological systems supports this notion, since Shh and Ihh appear to be closer in potency and function than Dhh in the majority of assays. It has been postulated that *Shh* has maintained essential ancestral roles in midline patterning, leaving *Ihh* and *Dhh* genes free to diverge and take on new roles (Kumar et al., 1996; Shimeld, 1999). Consistent with this is the observation that the *Shh* gene knockout in mice is embryonic lethal (Chiang et al., 1996), while the *Dhh* knockout animals survive to adulthood with only minor defects (Bitgood et al., 1996). Here we show that the three Hh proteins have the potential to function similarly, although in certain assays they have different potencies. These differences in the ability to elicit specific biological responses may reflect unknown control mechanisms for modulating Hh activity during development. Whether they represent the presence of additional unidentified factors on the pathway or result from sequence differences for Shh, Ihh, and Dhh remains to be determined.

4. Experimental procedures

4.1. Preparation of Hh

Recombinant human Shh (residues 24–197) was expressed in *E. coli* and purified as described previ usly (Pepinsky et al., 1998; Williams et al., 1999). cDNAs encoding human Ihh (residues 28–202) and human Dhh (residues 23–198) (Curis Inc., Cambridge, MA, USA) were subcloned into the pET11d expression vector (Novagen) as His-tagged constructs with an enterokinase cleavage site engineered into the constructs adjacent to the N-terminal cysteine, and transformants were grown as described previously for His-tagged human Shh (Williams et al., 1999). Ihh and Dhh were purified following the protocol described previously for the purification of human Shh (Williams et al., 1999).

4.2. Hedgehog cell-based assays

Ptc binding and C3H10T1/2 AP induction assays were performed as described previously (Pepinsky et al., 1998; Williams et al., 1999). For Ptc-binding experiments, EBNA 293 cells transiently transfected with a Myc-tagged, C-terminally truncated murine *ptc* cDNA construct (a gift of Matt Scott, Stanford University) were titrated with serial two-fold dilutions of each test compound in the presence of 5 nM Shh-IgG_1 fusion protein reporter (Shh-Fc) (Williams et al., 1999). Cells were washed, fixed, and read on a fluorescence-activated cell sorter. Binding constants were calculated from single determinants for each sample. Shh-Fc was observed to bind to Ptc directly with a K_D value of 3 nM. An assay to measure Hh binding to Hip was performed by transiently transfecting a *Hip* cDNA construct (a gift of Andy McMahon, Harvard University) into EBNA 293 cells and measuring Hh binding as described for the Ptc binding assay above. For this assay, Shh-Fc was observed to bind to Hip directly with a K_D value of 10 nM.

For assessing activity, C3H10T1/2 cells (ATCC) were incubated for 5 days with serial dilutions of each Hh protein. The cells were lysed and assayed for AP activity using the chromogenic substrate *p*-nitrophenyl phosphate (read at 405 nm). Each sample was analyzed in duplicate, and EC_{50} values were measured from the mean data values. Samples were also tested for function on C3H10T1/2 and TM3 cells (murine Leydig cell, ATCC) transfected with a luciferase reporter under the control of the *gli-1* promoter. Details of this assay will be published elsewhere (J.A. Porter, D. Bumcrot, and G.S.B. Horan, unpublished data).

The induction of *gli-1*, *ptc*, *ptc-2*, and *hip* mRNA by Hh in C3H10T1/2 and TM3 cells was measured by RT-PCR as described previously (Williams et al., 1999). Total RNA was extracted from the cells with Trizol (Life Technologies, Gaithersburg, MD, USA) and subjected to RT-PCR. Primer sequences for *gli-1* were: 5′-CGGGGTCTCAAACTGCCCAGCTT-3′, and 5′-GGCTGGGTCACTGGCCCTC-3′.

4.3. Chondrocyte differentiation assay

The ability of the different Hh proteins to block chondrocyte differentiation was assessed by culturing E16.5 mouse hindlimbs with different concentrations of human Shh, Ihh, or Dhh proteins. Hindlimbs were severed at mid-femur, deskinned, and cultured as described previously (Vortkamp et al., 1996). Treated hindlimbs were cultured with either 20, 10, 5, 1, or 0.5 μg/ml of Hh protein, while the contralateral

hindlimb was cultured as an untreated control. After 4 days in culture, limbs were fixed in 4% paraformaldehyde/PBS. The state f chondrocyte differentiation was compared by detecting *Collagen X* mRNA expression in the growth plate of the proximal tibia. In situ hybridizations (5 μm serial sections) were performed using ^{33}P-UTP-labeled riboprobes as described previously (Vortkamp et al., 1996). Hybridizations were performed at 70°C in 50% formamide, and post-hybridization washes were carried out at 55°C in 50% formamide/2 × SSC. For serial sections, three successive sections were collected.

4.4. Left/right assay

Hamburger–Hamilton (HH) stage 4 chick embryos were processed for New culture (New, 1955) and implanted with protein-soaked beads as described previously (Levin et al., 1995). Affigel-Blue beads were soaked in either 1, 0.5, or 0.1 mg/ml of Hh protein. Protein-soaked beads were implanted on the right side of Hensen's Node. Embryos were harvested at stage 8, fixed in 4% paraformaldehyde/PBS, and processed for whole-mount in situ hybridization using a probe for *nodal*, as described previously (Levin et al., 1995).

4.5. Digit duplication assay

Fertilized chicken eggs (Spafas) were incubated at 38°C until the embryos reached HH stage 21. Protein-soaked beads were then placed in the anterior region of the right-side wing bud as described previously (Riddle et al., 1993). Beads were soaked in either 8, 2, or 0.5 mg/ml of Hh protein. Embryos were harvested at stage 36, fixed in 4% paraformaldehyde/PBS, and stained with Alcian blue. Polarizing activity was assessed by scoring for the degree of digit duplication.

4.6. Neural plate explant assay

The motor neuron-inducing activity of the Hh proteins was assessed in a neural plate explant assay as described previously for human Shh (Williams et al., 1999). Intermediate neural plate explants dissected from the caudal region of stage 10 chick embryos were embedded in a collagen gel, Hh protein was added, and the explants were cultured for 60 h in N2 medium. After fixing with 1% paraformaldehyde, Islet-1-positive nuclei were detected with an anti-Islet-1 mAb (Developmental Studies Hybridoma Bank, University of Iowa; deposited by Dr T. Jessell, Columbia University) and counted using a microscope equipped with fluorescence optics.

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